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The potential of antibiotics, chemical biocides and lytic enzymes in preserving nutrients, biological oxygen demand and oil and grease in water and sewage effluents was studied. Preliminary studies concerning the effect of drugs on cell growth and oxygen utilization in samples stored at room temperature led to the selection of chlorhexidine, vantocil and many combinations each containing three antibiotics from among polymyxin B, neomycin, erythromycin, streptomycin and chloramphenicol. The effective concentration range was ~50 µg/ml each antibiotic for clean waters and as high as 200 µg/ml each antibiotic for heavily polluted water (e.g. primary effluents).

Chlorhexidine and vantocil IB stabilized nitrate and nitrite in fresh waters and relatively clean secondary effluents only. Presence of antibiotics caused interference in determination of orthophosphate. The antimicrobial agents tested interfered with BOD determination by causing inhibition of oxygen depletion and therefore were of no value for preservation of this parameter. Efforts to selectively remove and/or inactivate the drug before BOD determination were unsuccessful. Oil and grease levels were stabilized by antibiotics for up to two weeks in relatively clean waters only. The results demonstrate that antibiotics offer a viable alternative to conventional methods for preservation of some constituents and parameters in fresh water samples but not in sewage effluents.

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NEW APPROACHES TO THE PRESERVATION OF CONTAMINANTS IN WATER SAMPLES

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FOREWORD

Environmental measurements are required to determine the quality of ambient water and the character of waste effluents. The Environmental Monitoring and Support Laboratory - Cincinnati conducts research to:

- Develop and evaluate techniques to measure the presence and concentration of physical, chemical and radiological pollutants in water, wastewater, bottom sediments, and solid waste.

- Investigate methods for the concentration, recovery, and identification of viruses, bacteria, and other microbiological organisms in water; and to determine the responses of aquatic organisms to water quality.

- Develop and operate an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.

There is an ever-increasing interest to maintain sample integrity during the sample collection-sample analyses cycle. Present preservation techniques have limitations. Biological reactions taking place in a sample may cause a breakdown of contaminants, a conversion of soluble constituents to organically bound material in cell structures, a solubilization of insoluble material, or even the conversion of material into a gaseous phase which then escapes from the sample. In view of the limitations of the preservation methods currently used, this report looks at the preservation possibilities in the biological control of microorganisms through the use of enzymes and antibiotics.

Dwight G. Ballinger, Director
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Laboratory - Cincinnati

ABSTRACT

The potential of antibiotics, chemical biocides and lytic enzymes in preserving nitrogen and phosphorus series of nutrients, biological oxygen demand, and oil and grease in water and wastewaters was studied. Preliminary selection of agents for sample preservation was based on their ability to inhibit cell growth and oxygen utilization in primary and secondary effluents and natural water samples. The drugs which exhibited potential based on this criteria were: chlorhexidine, vantocil IB, polymyxin B + neomycin + streptomycin (PNS), erythromycin + polymyxin B + neomycin (EPN), erythromycin + polymyxin B + streptomycin (EPS), polymyxin B + chloramphenical + neomycin (PCN) and gentamycin + amikacin + polymyxin B (GAP). The effective concentration range was 100 - 200 µg/ml for primary effluents, 50 - 100 µg/ml for secondary effluents, and 10 - 50 µg/ml for fresh waters. Lysozmyme with or without ethylenediamine tetraacetic acid or Tris failed to control microorganisms in wastewater samples. Sodium sulfide, which has been used to slow the growth rate of bacteria in suspension, was ineffective as well.

Chlorhexidine and vantocil IB stabilized nitrate and nitrite in fresh water and relatively clean secondary effluents. Other antibiotics were unsuccessful in preservation of these nitrogen forms. Antibiotic mixtures with erythromycin as one component interfered with NO_3 determination. Presence of antibiotics caused interference in determination of inorganic phosphate by vanadophosphoric or ascorbic acid methods.

The antimicrobial agents tested did not offer a practical solution to the preservation of BOD in wastewaters due to the fact that the agents added for preservation inhibited the activity of the seed even after the dilutions required for BOD determination. Efforts to selectively remove and/or inactivate the test antimicrobial agent prior to BOD determination by boiling the sample in a sealed ampoule, or pH adjustment, were unsuccessful. Other approaches tested including preacclimation of seed to the test antimicrobial agent and the use of lower concentrations of the preservation also failed to relieve the inhibition.

Oil and grease levels were stabilized by GAP and PCN for up to 2 weeks in relatively clean waters only. Chlorhexidine and vantocil IB effectively stabilized pH and dissolved oxygen in natural water samples but were not effective in municipal wastewaters.

The results demonstrate that antibiotics offer a viable alternative to conventional methods for preservation of fresh water samples but not for preservation of sewage effluents.

This report is submitted in fulfillment of Grant R 804609 by Syracuse Research Corporation under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from 9-1-1976 to 11-30-1978 and work was completed as of 7-1-1978.

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ABBREVIATIONS AND SYMBOLS

µg/ml	micrograms/milliliter
mg/ℓ	milligrams/liter
ppm	parts per million
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
TKN	Total Kjeldahl Nitrogen
D.O.	Dissolved Oxygen
APHA	American Public Health Association
Tris	Tris(hydroxymethyl)aminomethane
EDTA	Ethylenediaminetetracetic acid

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SECTION 1

INTRODUCTION

Increased effort to prevent water pollution has led to higher demands for analysis of waste before discharge, and receiving waters. For example, analysis of municipal wastewaters for selected constituents and parameters is required by regulatory agencies for NPDES permit program. The number of analyses that can be performed at the sampling site are very limited, and samples must be transported or shipped to a laboratory for detailed chemical analysis.

A sample of polluted water is an extremely complex and dynamic system containing many chemical and biological entities. Since the majority of the water monitoring sites are at remote locations where "grab" samples are taken for further chemical analyses, the microbial utilization or transformation of some chemical constituents in a water sample enroute to, or in storage at, a laboratory may be a source of considerable error, especially in trace analysis of pollutants. In order to have correct information on the concentration of contaminants in the water samples, it is necessary to stop the changes that take place in the samples after their collection.

The preservation methods available at present - pH control, chemical addition, refrigeration, and freezing have many limitations, either mechanical or chemical. These methods are based on some of the very early work on samples preservation. For instance, the recommended method to stabilize phenols is a modification of the work performed over 30 years ago (Ettinger et al., 1943). The basic problem is that many of these preservation systems do not work well. Certain chemical preservatives interfere with analytical methods or may significantly alter the chemical composition of the sample. Preservation of samples by refrigeration or freezing is impractical and expensive. When $HgCl_2$ is used as an antimicrobial agent, the disposal of the mercury containing sample is a recognized problem.

In view of the limitations of the preservation methods currently in use, this investigation was undertaken to test the potential of antibiotics, chemical biocides, and lytic enzymes for preserving water samples. Such untried systems may offer a distinct improvement over existing procedures. Since many alterations in water and wastewaters samples are produced by microbial activity, efforts in this study were directed towards controlling these alterations as opposed to chemical and physical alterations and losses.

The study was divided into two phases. The first phase examined the effect of numerous antimicrobial agents on the microbial population in waters of varying purity with the objective of selecting agents with potential for

sample preservation, and the determination of effective concentration ranges for such agents. The second phase involved studying the preservation of contaminants and sample parameters by the antimicrobial agents selected in phase 1.

SECTION II

GENERAL CONCLUSIONS

Antibiotics and biocides tested demonstrate potential for stabilization of sample constituents and parameters in fresh waters. However, their utilization for preservation of sewage samples is unlikely because of the diversity of microorganisms present.

Chlorhexidine and vantocil IB can be used to stabilize nitrate and nitrite in fresh waters for at least 16 days at room temperature. They can not be used for preservation of phosphates because of interference with the analytical method for phosphates.

Preservation of water and wastewater samples with antimicrobial agents for BOD is not feasible because of the interference caused by the preservatives in biological activity during measurement. At lower concentrations, the antimicrobial agents used tend to be utilized as carbon sources by microorganisms resulting in erroneous BOD values. Oil and grease can be statilized for up to two weeks in relatively clean effluents with the addition of GAP and PCN.

The study has not led to a suitable and practical method of water and wastewater sample preservation and efforts in this direction should be continued.

SECTION III

RECOMMENDATIONS

A preservative must satisfy many requirements before it can be regarded as suitable for water and wastewater sample preservation. General requirements which must be met are (i) it should be more effective than the methods presently available, (ii) it should stabilize a large number of sample constituents/parameters, (iii) it should offer no or minimal interference with the analytical procedure, and (iv) it should be safe to use and pollution-free. Apparently, the process of finding a suitable preservation method is expected to be slow and time consuming.

The studies undertaken have not led to a practical solution to the problem of water and wastewater samples preservation, and hence the search for new methods of sample preservation must be continued. The approaches which have not been successful in our studies should provide clues to those methods which will be successful.

It is recommended that research be pursued in the following avenues:

1) Preservation of sewage effluents should be attempted with other untried antimicrobial agents. For hard to preserve sample constituents/parameters, the antibiotic action should be used in conjunction with other methods e.g. sample storage at 4°C, preheating of the sample, pH adjustment, etc.

2) Efforts should be directed towards identifying the species of microorganisms which show resistance to antimicrobial agents. This will help in devising methods to control them. A recent paper by Kelch and Lee (1978) entitled "Antibiotic resistance pattern of gram negative bacteria isolated from environmental sources" provides some information in this direction.

3) Antibiotics and biocides show promise for preservation of fresh water samples and should be investigated further.

4) The use of antimicrobial agents for preservation of BOD in water and wastewater samples does not appear feasible and it is recommended that other approaches be explored. Examples of approaches which could be tried are: gamma irradiation, quick heating by microwave, etc.

SECTION IV

LITERATURE SEARCH

During the initial phase of the project, a literature search was conducted to obtain relevant references relating to the general subject of water sample preservation. Papers were gathered with the aid of computerized and manual searches. The key words selected were: water storage, preservation, contaminants, water sample, urine, stability, oil and grease, total organic carbon, chemical oxygen demand, biological oxygen demand, nitrate, phosphate and phenol. Back issues of the Journal of Antimicrobial Agents and Chemotherarpy were searched to collect information regarding recently developed antimicrobial agents. The result of the review work is reflected by the different pertinent references cited in various sections of the report.

NTIS and SSIE data bases were searched in order to obtain information concerning other on-going or recently completed projects in the area of water sample preservation. The search revealed no relevant projects.

SECTION V

BACKGROUND

This section presents information concerning alteration of water and wastewater composition during storage and/or transportation, and the methods available to prevent these changes. A general description is given here to form a basis for the study undertaken. Information pertinent to the stabilization of specific sample constituents and parameters studied is presented in respective sections later in the report.

STABILITY OF WATER POLLUTANTS DURING STORAGE AND/OR TRANSPORTATION

The stability of the water samples in transit from the sampling site to the laboratory, or during storage, has received very little attention. Alteration in the concentration of contaminants in the water samples can generally be attributed to (i) biological transformation, (ii) alteration by chemical agents (hydrolysis, oxidation, etc.) and (iii) physical losses (adsorption, volatilization, etc.).

The available information suggests that biological agents, particularly bacteria, play a dominant role in causing these changes. It has become well established that, when fresh water or seawater is stored in glass containers, bacteria multiply rapidly to numbers which are often far in excess of those found under natural conditions (Franklands, 1894; Whipple 1901; ZoBell and Grant, 1943; ZoBell, 1946; Taylor and Collins, 1949). The growth of bacteria in waters transferred to containers is rapid, and maximum numbers are usually found on the second or third day after filling (ZoBell and Stadler, 1940). This growth is apparently at the expense of the contaminants present in the water samples. The biological transformation reactions summarized in Table 1 are examples of the biological influence on sample composition. Although all the changes listed in Table 1 have not been directly shown to occur in water samples, such alterations have been noted in the environment. It is likely that the microorganisms responsible for these conversions will also be present in natural waters.

Phenol in moderate concentration has been reported to be degraded by microorganisms very rapidly (Harlow, 1939, Ettinger *et al.*, 1943). The biochemical activity has been reported at pH values ranging from low of 2 (Ruchhoft *et al.*, 1940) to high of 13 (Theriault and McNamee, 1930). A positive correlation between the loss of phenolic compounds and microbiological activity in wastewaters has been noted by Carter and Huston (1978). Ettinger *et al.* (1943) reported over a 90% loss of the phenol content of a

TABLE 1. EXAMPLES OF THE REACTIONS WHICH MAY BE RESPONSIBLE FOR CONTAMINANT CONCENTRATION CHANGES IN WATER SAMPLES

Pollutant	Example of microorganisms which may be responsible for pollutant concentration changes *	Chemical or physical alterations affecting pollutant concentration	Nature of changes produced	Reference
Inorganic nitrogen (Ammonia, nitrate & nitrite)	Bacillus mycoides, Proteus vulgaris, E. Coli		nitrogenous organic matter → Amino acids → NH_3	Thimann, 1963
	Nitrosomonas		$NH_4^+ \rightarrow NO_2^-$	Aleem, 1970
	Nitrobacter		$NO_2^- \rightarrow NO_3^-$	Aleem, 1970
Sulfide & sulfate	Proteus vulgaris		cysteine / Methionine $\rightarrow H_2S\uparrow$	Thimann, 1963
	Thiobacilli		$H_2S \rightarrow S^o \rightarrow S_2O_3^= \rightarrow SO_4^=$	Saxena, 1970
	Desulfovibrio		$SO_4^= \rightarrow H_2S\uparrow$	Thimann, 1963
Sulfide		acid pH	$H_2S\uparrow$	
Organic nitrogen	Bacillus mycoides, Proteus vulgaris, E. Coli		organic nitrogenous compounds → NH_3	Thimann, 1963
Phosphate	all microorganisms		Inorganic phosphate ↔ organic phosphate	Thimann, 1963
Herbicides				
Phenylureas	Xanthomonas sp. Sarcina sp. Bacillus sp. Pseudomonas sp.		Used as carbon source	Kearney, 1966
Phenylcarbamates	Pseudomonas sp. Flavobacterium sp. Agrobacterium sp. Acromobacter sp.		Used as carbon source	Kearney, 1966
Chlorinated aliphatic acids	Arthrobacter sp.		Used as carbon source	Kearney, 1966
Phenoxyalkonoic acids	Nocardia, Flavobacterium sp.		Used as carbon source	Kearney, 1966; Goerlitz and Lamar, 1967
Phenoxyalkonoic acid esters		Hydrolysis	Ester → acid	Goerlitz and Lamar, 1967
Insecticides				
Dieldrin	Pseudomonas sp. Bacillus sp.	adsorption on container wall	Unidentified	Menzia, 1969 Weil and Quentin, 1970
Detergents				
Alkyl benzene sulfonate	Pseudomonas sp.		Alkyl benzene sulfonate ↓ Inorganic sulfur compounds, tertiary butyl alcohol, phenol, benzoic acid etc.	Horvath and Koft, 1972
Hydrocarbons	Pseudomonas sp., Micrococcus sp., Mycobaterium sp., Nocardia		Several hydrocarbons serve as source of carbon for growth	McKenna and Kallio, 1965
Other aliphatic and aromatic compounds (including phenolics)	Acetobacter sp. Pseudomonas sp.		Most are used as carbon source	Heukelekian and Dondero, 1963; Stanier and Doudoroff, 1970; Thimann, 1963.

* Although these microorganisms have been isolated mostly from soil, similar microorganisms can be expected to be present in the aquatic environment, since the bacterial population of water depends predominantly on the extent and character of its contact with earth's surface.

Mahoning River sample when stored as collected. A reduction in the phenol content of the river water sample was also observed during storage at 2°C; under these conditions nearly 90% of the initial phenol present was lost in 11 days. Rapid breakdown of monochlorophenols in polluted surface waters has been reported by Ettinger et al. (1950). The change in concentration of some chlorinated aliphatic hydrocarbons (chloroform, 1,1,2-trichloroethane, and 1,1,2,2-tetrachloroethane) in seawater and sterilized fresh water was examined by Jensen and Rosenberg (1975). Four different systems were used: (1) daylight, open system, (2) daylight, closed system, (3) darkness, closed system, (4) darkness, 2 atm. closed system. The disappearance of the compounds was greatest in the open aquaria, indicating that loss due to evaporation was greater than other degradation losses. The decrease in certain chlorinated aliphatic hydrocarbons was also attributed to biological degradation.

Dokiya et al. (1974) noted that organomercurial compounds were lost from natural marine water in 15 days, but not from distilled water. This suggested that perhaps microorganisms present in the marine water may have been responsible for the loss. Swisher et al. (1973) have reported biodegradation of nitrolotriacetate-metal chelates in river water upon incubation.

The studies described above clearly show that a considerable change in the composition of a water sample may take place during storage and transportation. The instability of some chemical constituents in water samples may be a considerable source of error in their analysis.

AVAILABLE METHODS OF PRESERVATION OF WATER SAMPLES, AND THEIR LIMITATIONS

Most water samples for organic and inorganic analysis must be protected from changes in composition from the time of collection to the time they are analyzed in the laboratory (i.e. during transport and storage of the sample). The methods currently used by the Environmental Protection Agency to stabilize contaminants in water samples are pH control, chemical addition and refrigeration (U.S.E.P.A., 1974). These methods are intended to control biological activity, retard hydrolysis and complex formation, and reduce volatility of constituents. Refrigeration is the most acceptable method for controlling microorganisms since it inhibits their growth without otherwise altering the sample. However, the technique is difficult to use in field settings. Sulfuric acid has generally been used as a bacterial inhibitor in samples for COD, oil and grease, organic carbon, etc. Presently, a combination of refrigeration and sulfuric acid is used to preserve oil and grease in water samples. The type of preservation methods as they relate to the various water parameters are presented in Table 2.

Antibiotics and biocides as substitutes for the conventional methods of preserving water and wastewater samples have received little attention, even though they offer many advantages over the existing methods. For example, unlike $HgCl_2$, antibiotics are pollution free and safe to handle. Berg et al. (1966) reported stabilizing COD in primary effluents by heating the effluent to 80°C and subsequent addition of polymyxin B. Heating alone failed to control microbial population in these samples. The potential of antibiotics and biocides for inhibiton of microbial activity in samples is also

TABLE 2. EXAMPLES OF PRESERVATION TECHNIQUES CURRENTLY IN USE
(U.S.E.P.A., 1974, Huibregtse and Moser, 1976)

Parameter	Preservative	Maximum holding period
Acidity-Alkalinity	Refrigeration at 4°C	24 hours
Biochemical Oxygen Demand	Refrigeration at 4°C	6 hours
Chemical Oxygen Demand	H_2SO_4 to pH <2	7 days
Chloride	None required	7 days
Color	Refrigeration at 4°C	24 hours
Cyanide	NaOH to pH 12	24 hours
Dissolved Oxygen	Determine on site	No holding
Herbicides	Acidify samples to pH 4.0	--
Metals, Total	5 ml HNO_3 per liter	6 months
Nitrogen, Ammonia	Cool, 4°C H_2SO_4 to pH <2	24 hours
Nitrogen, Kjeldahl	Cool, 4°C H_2SO_4 to pH <2	24 hours
Nitrogen, Nitrate	Cool, 4°C H_2SO_4 to pH <2	24 hours
Nitrogen, Nitrite	Cool, 4°C	24 hours
Oil and Grease	H_2SO_4 to pH <2, Cool, 4°C	24 hours
Organic Carbon	H_2SO_4 to pH <2	24 hours
pH	Determine on site	No holding
Phenolics	1.0 g $CuSO_4/\ell$ + H_3PO_4 to pH <4 Cool, 4°C	24 hours
Surfactants	Cool, 4°C	24 hours
Total Phosphorus	Cool, 4°C	24 hours

revealed by the studies undertaken by Collins et al. (1976). These authors reported that nitrification in aquatic recirculation systems could be effectively inhibited by erythromycin or 2-chloro-6-(trichloromethyl)pyridine.

The diversity of the microorganisms present in the water samples makes complete and unequivocal preservation rather difficult. Unlike the sample compositional changes brought about by the presence of microorganisms, the changes caused by certain physical and chemical agents can rather easily be controlled. For example, by tightly stoppering the water sample, the losses due to volatilization can be reduced. By rinsing the sample bottle with the extraction solvent, the compound can be desorbed from the walls of the container (Breidenback et al., 1964). On the other hand, the methods available currently to prevent microbial activity in the samples are not very effective. Certain bacterial acidophiles will continue to grow in samples even if the pH is less than 2.0. Addition of 40 mg/ℓ $HgCl_2$, the recommended concentration for preservation, is often not completely effective in eliminating microbial growth in highly polluted samples, yet higher concentrations would present both environmental and health hazards (Huibregtse and Moser, 1976; Zobell and Brown, 1944). Storage at 4°C is not possible for long since microbial multiplication and metabolism will continue at this temperature, although at a slower rate than at ambient temperatures.

Many of the preservation systems produce extensive changes in the sample which may preclude certain analytical determinations. Availability of suitable method(s) of sample preservation is becoming increasingly important as more and more organic contaminants are being detected and monitored in water samples. Preservation of samples by refrigeration is free from many limitations but is expensive and difficult to effect in the field.

In view of the limitations of the preservation methods currently in use, the proposed investigation was intended to examine the potential of antibiotics, chemical biocides and lytic enzymes in sample preservation.

SECTION VI

PRELIMINARY SELECTION OF PRESERVATION AGENTS BASED ON THEIR ANTIMICROBIAL ACTION IN WATERS AND WASTEWATERS

The proposed preservation approaches were first screened by studying their effect on microorganisms in waters of varying degree of purity. Evaluation based on the effect on microbial growth and/or metabolic activity is simple and less time consuming than measurement of sample parameters/ constituents and hence can be applied to a large number of test agents. Moreover, it is a valid approach due to a direct correlation between microbiological activity and sample deterioration. The preservation systems which effectively controlled microbial growth and metabolic activity were pursued further. The effective concentration range of the preservants was also determined from these studies.

SAMPLES AND SAMPLING PROCEDURE

Tests were performed utilizing waters of varying degree of purity. Fresh waters with low levels of contamination were considered representative of relatively pure water, secondary effluents from activated sludge and the trickling filter process as moderately polluted water, and primary effluent as grossly polluted water.

Fresh water samples were obtained from two sources - Keuka Lake in Hammondsport, N.Y., and Chittenango Creek in Chittenango, N.Y. Keuka Lake samples were obtained from the "raw water" tap which serves as a source of water for the Dept. of Public Works in the Village of Hammondsport. The lake receives agricultural run-off and winery waste. Chittenango Creek samples were obtained from a location 2.5 miles south of the Village of Chittenango. The creek is a tributary of Oneida Lake and drains an area of 314 sq. miles. It receives discharge from a domestic wastewater treatment plant.

Secondary effluents used in these studies were obtained from (a) Wetzel Road Sewage Treatment Plant, Liverpool, N.Y., which uses trickling filters as a secondary treatment process, and (b) Meadowbrook-Limestone Wastewater Treatment Plant, Manlius, N.Y., which uses activated sludge as a secondary treatment process. Samples were obtained from the overflow weirs of the clarifiers. Primary effluent samples were obtained from the overflow weirs of the primary clarifier from Wetzel Road plant only.

Fresh samples were collected for each study and brought back to the laboratory within one hour. Tests revealed that storage of sample by freezing for use in later experiments was not feasible, since it resulted in

increased sensitivity of microorganisms towards drugs. Samples were brought to room temperature by warming in a water bath prior to use.

CHEMICAL AGENTS TESTED

Test compounds evaluated for their antimicrobial potential included antibiotics, chemical biocides and lytic enzymes. Sodium sulfide, a product found to be effective in rendering bacteria dormant in liquid suspension for indefinite periods (Horsfall and Gilbert, 1976) was also included in the study. The chemical nature and source of the antimicrobial agents tested are given in Table 3. Antibiotics were tested in combinations which were made based on information in the literature concerning the type of microorganisms controlled by specific antibiotics (Table 4). The objective was to achieve a broad spectrum mixture. The failure of single antibiotics to control microorganisms in water and wastewater samples revealed by preliminary experiments, prompted utilization of this approach. Stock solutions of antimicrobial agents were prepared in sterile distilled water and stored in refrigerator. Solutions of antimicrobial agents which were known to be unstable under these conditions were prepared fresh for each experiment.

EXPERIMENTAL PROCEDURE

The water samples under investigation were homogenized by shaking and aliquoted into BOD bottles containing a predetermined volume of the test preservative solution. Untreated samples served as control. The bottles were incubated at room temperature. At specific time points, dissolved oxygen was measured by immersing a BOD probe in the bottles and 1 ml aliquots were withdrawn for standard plate count. Media preparation, dilution, plating and counting was performed according to the procedure described in APHA Standard Methods (APHA, 1975). Plate counts were done in triplicate and the results expressed as an average of the three measurements. In some experiments the results are expressed as less than a fixed number of colonies/ml, for example $<10^3$. This fixed number represents the lowest dilution of sample plated and was chosen in such a way as to sufficiently dilute the antibiotics to remove any growth inhibition yet enable enough colony growth on a plate to make the count statistically significant. The time course for most experiments described in this section was 4-6 days. This incubation period was considered adequate for the following reasons: (i) microbial growth was complete during this time in untreated samples, therefore, the effect of drug treatment on all phases of the growth cycle could be studied, (ii) in instances where microorganisms overcame the inhibitory effect of drugs, this phenomenon occurred between 24-48 hours and hence the incubation period was sufficient to allow us to note the ineffectiveness of the drug, and (iii) the aim in examining the antimicrobial action of drugs was only to make a preliminary selection for detailed sample preservation studies.

TABLE 3. CHEMICAL NATURE AND SOURCE OF THE ANTIMICROBIAL AGENTS STUDIED

Antibiotics - common name	Chemical nature*	Source
Amikacin	semisynthetic aminoglycoside antibiotic	Bristol Labs
Amoxicillin	β-lactam antibiotic	Bercham Lab.
Ampicillin	modified penicillin antibiotic	Bristol Labs
BL-5786	semisynthetic cephalosporin	Bristol labs
Chloramphenicol		Sigma Chem. Co.
Chlortetracycline hydrochloride	tetracycline antibiotic	Sigma Chem. Co.
Polymyxin E	polypeptide antibiotic	Sigma Chem. Co.
Erythromycin	macrolide antibiotic	Sigma Chem. Co.
Gentamicin	aminoglycoside antibiotic	Sigma Chem. Co.
Mecillinam	β-lactam antibiotic	Hoffmann-LaRoche, Inc.
Minocycline	tetracycline analog	Lederle Labs
Neomycin Sulfate	aminoglycoside, antibiotic	Sigma Chem. Co.
Oxytetracycline dihydrate	tetracycline antibiotic	Sigma Chem. Co.
Polymyxin B Sulfate	polypeptide antibiotic	Sigma Chem. Co.
Streptomycin	aminoglycoside antibiotic	Sigma Chem. Co.
Tetracycline	tetracycline antibiotic	Sigma Chem. Co.
Ticarcillin		Bercham Lab
Biocides		
Cetrimide	cationic antiseptic (cetyltrimethyl ammonium biomide)	Sigma Chem. Co.
Chlorhexidine	substituted biguanide	ICI, Ltd.
Vantocil IB	substituted biguanide	ICI Ltd.
Positive Controls		
Mercuric Chloride ($HgCl_2$)		Fisher Scientific
Enzymes		
Lysozyme	Muramidase-enzyme isolated from chicken egg white	ICN
Miscellaneous agents		
Sodium Sulfide		Fisher Scientific

* From Korzybski et al., 1967; Lorian, 1966.

TABLE 4. ANTIMICROBIAL AGENTS TESTED FOR THEIR EFFECT ON MICROBIAL POPULATION IN WATERS- AND WASTEWATER SAMPLES

Test agent	Referred to in the text as *	Test concentration range (µg/ml each drug)
Chlorhexidine	Chlorhexidine	50 - 200
Vantocil IB	Vantocil	50 - 100
Amikacin + Gentamicin	--	100 - 200
Amikacin + Polymyxin	--	100 - 200
Amoxicillin + Mecillinam	--	100 - 200
BL-S786 + Neomycin	--	100 - 200
Neomycin + Colistin	--	100 - 200
Polymyxin + Neomycin	PN	100 - 500
Polymyxin + Gentamicin	--	50 - 100
Polymyxin + Ticarcillin	--	100 - 200
Amikacin + Amoxicillin + Polymyxin	--	100 - 200
Ampicillin + Mecillinam + Neomycin	--	100 - 200
Ampicillin + Streptomycin + Neomycin	--	100 - 200
Gentamicin + Amikacin + Polymyxin	GAP	100 - 200
Neomycin + Ticarcillin + Polymyxin	--	100 - 200
Neomycin + Streptomycin + Colistin	--	100 - 200
Neomycin + Mecillinam + Amoxicillin	--	100
Polymyxin + Neomycin + Streptomycin	PNS	50 - 100
Polymyxin + Neomycin + Ampicillin	PNA	100 - 200
Erythromycin + Polymyxin + Neomycin	EPN	100 - 200
Erythromycin + Polymyxin + Streptomycin	EPS	100 - 200
Polymyxin + Chloramphenicol + Neomycin	PCN	100 - 200

*Abbreviations are given only for those drugs which are repeated in the text.

RESULT AND DISCUSSION

Effect of Drugs on Microorganisms in Sewage Samples

The effect of selected drugs on viable cell count and oxygen utilization in primary and secondary effluents stored in glass bottles is shown in Figs. 1-4. Data is not shown for the drugs which were without effect. These drugs were amikacin + gentamycin, amikacin + polymyxin, amoxicillin + mecillinam, BL-S786 + neomycin, neomycin + colistin, polymyxin + gentamycin, polymyxin + streptomycin + neomycin, neomycin + tricarcillin + polymyxin, neomycin + streptomycin + colistin, and neomycin + mecillinam + amoxicillin. Complete control of microorganisms* in primary effluents could not be achieved with any of the drugs tested except for chlorhexidine in one sample. Vantocil, EPN and EPS partially inhibited microorganisms in these samples. Oxygen utilization data shown in Fig. 4 corroborated these findings to some extent. It was surprising that PN and PNS caused partial inhibition of oxygen utilization, although they failed to inhibit growth. Some inconsistencies in the results were noted between the effluent samples collected on different dates. For example, EPN controlled cell numbers in effluents collected on 3-29-1978 but not in those collected on 6-27-1978. The failure of the drug may be linked to higher initial cell populations in the latter sample, or other differences between the two samples.

In secondary trickling filter effluents, biocides, vantocil IB and chlorhexidine proved very effective in controlling microbial population and the oxygen utilization data confirmed their effectiveness (Fig. 4). Antibiotic combinations - PCN, EPN, PNA, EPS and PNS - succeeded in keeping the cell population low for 24 hours, but growth resumed afterwards. These biocides and antibiotic combinations at 100 µg/ml (each antibiotic) were without effect in the secondary effluents from activated sludge process. Strong inhibition of microbial growth was noted with some drugs in these effluents when concentration was doubled (Fig. 3).

Studies were performed with antibiotic combinations - EPN, EPS and PN (100 µg/ml each antibiotic) - to determine if divided applications of the antibiotics over a period of time were more effective than a single application in the beginning. The results showed no significant increase in effectiveness of drugs when one-half the amount of the drug was added in the beginning and the remaining half at 24 or 48 hours.

Commercially available egg white lysozyme failed to control microorganisms in wastewater samples. In fact, the presence of lysozyme caused stimulation of growth which may be linked to the ability of some microorganisms to utilize lysozyme as a nutrient source. The predominance of Gram-negative Enterobacteria in sewage effluents may have been responsible for the ineffectiveness of lysozyme. Addition of EDTA and/or Tris, a treatment which is

* The term "microorganisms" is used here to describe all the organisms capable of forming a colony on standard plate count agar.

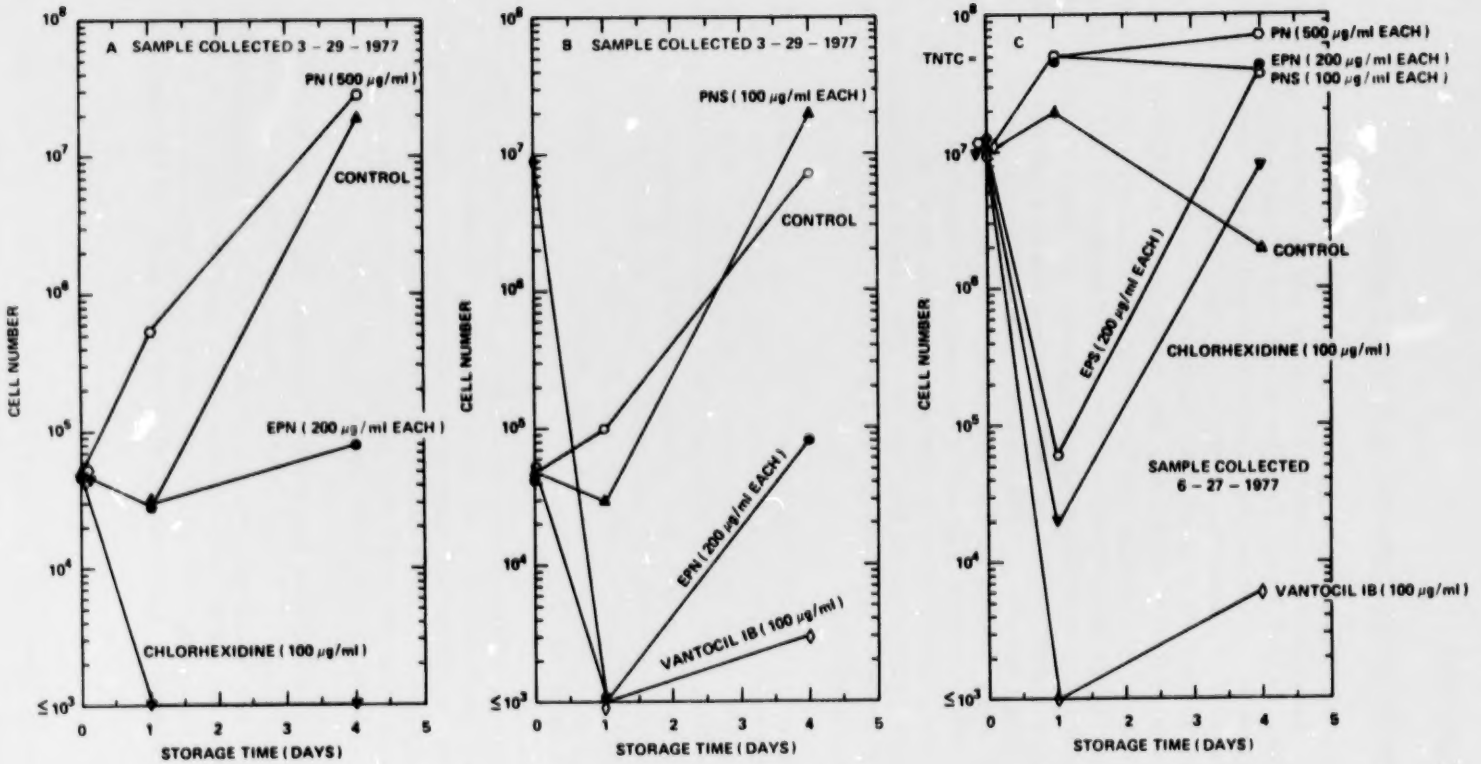
A SAMPLE COLLECTED 3 – 29 – 1977
PN (500 µg/ml)
CONTROL
EPN (200 µg/ml EACH)
CHLORHEXIDINE (100 µg/ml)
CELL NUMBER
STORAGE TIME (DAYS)
B SAMPLE COLLECTED 3 – 29 – 1977
PNS (100 µg/ml EACH)
CONTROL
EPN (200 µg/ml EACH)
VANTOCIL IB (100 µg/ml)
CELL NUMBER
STORAGE TIME (DAYS)
TNTC =
C
PN (500 µg/ml EACH)
EPN (200 µg/ml EACH)
PNS (100 µg/ml EACH)
CONTROL
EPS (200 µg/ml EACH)
CHLORHEXIDINE (100 µg/ml)
SAMPLE COLLECTED
6 – 27 – 1977
VANTOCIL IB (100 µg/ml)
CELL NUMBER
STORAGE TIME (DAYS)

Figure 1. Effect of test agents on microbial population in primary municipal effluents collected at two different dates.

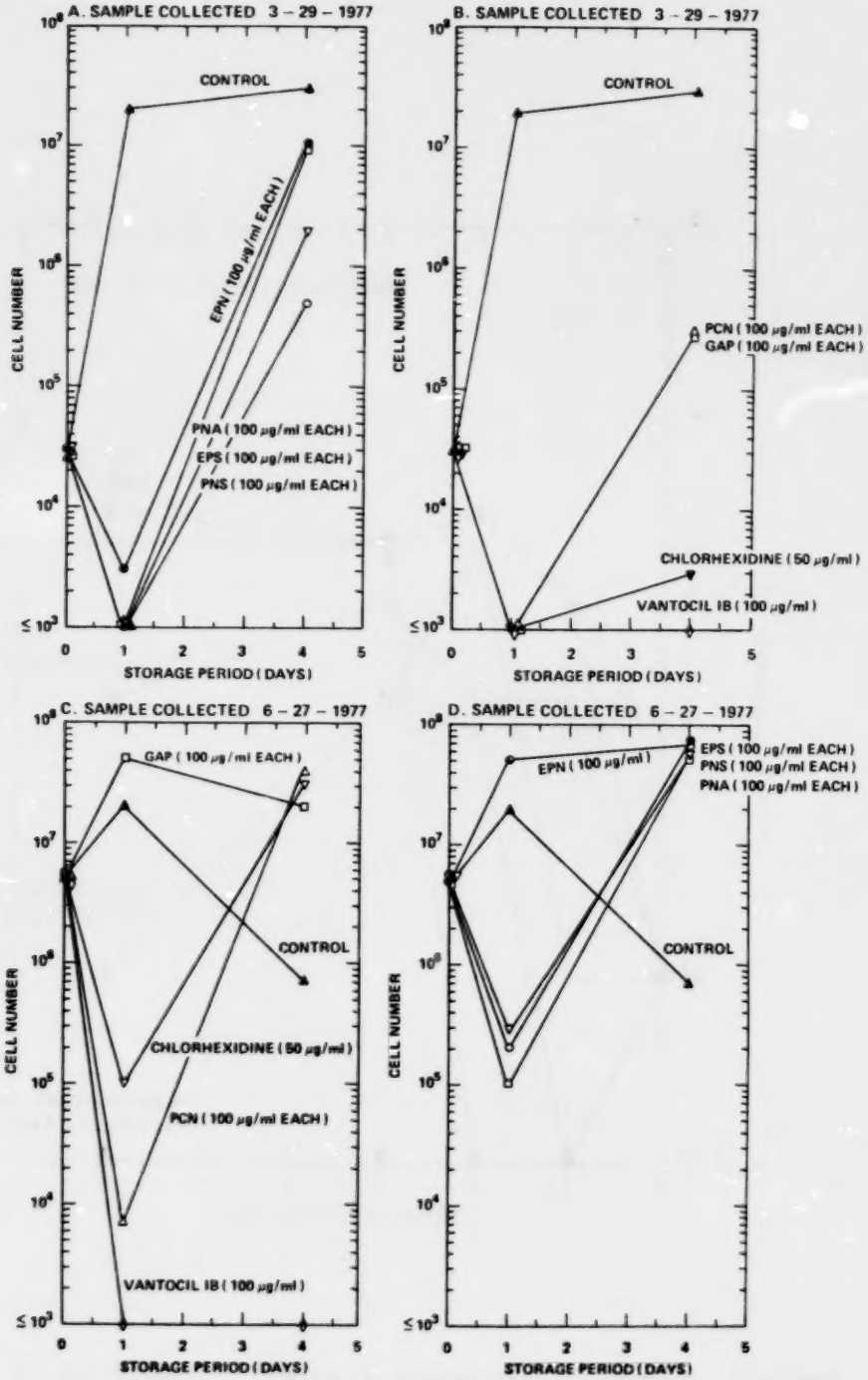
A. SAMPLE COLLECTED 3 – 29 – 1977
CONTROL
EPN (100 µg/ml EACH)
PNA (100 µg/ml EACH)
EPS (100 µg/ml EACH)
PNS (100 µg/ml EACH)
CELL NUMBER
STORAGE PERIOD (DAYS)
B. SAMPLE COLLECTED 3 – 29 – 1977
CONTROL
PCN (100 µg/ml EACH)
GAP (100 µg/ml EACH)
CHLORHEXIDINE (50 µg/ml)
VANTOCIL IB (100 µg/ml)
CELL NUMBER
STORAGE PERIOD (DAYS)
C. SAMPLE COLLECTED 6 – 27 – 1977
GAP (100 µg/ml EACH)
CONTROL
CHLORHEXIDINE (50 µg/ml)
PCN (100 µg/ml EACH)
VANTOCIL IB (100 µg/ml)
CELL NUMBER
STORAGE PERIOD (DAYS)
D. SAMPLE COLLECTED 6 – 27 – 1977
EPN (100 µg/ml)
EPS (100 µg/ml EACH)
PNS (100 µg/ml EACH)
PNA (100 µg/ml EACH)
CONTROL
CELL NUMBER
STORAGE PERIOD (DAYS)

Figure 2. Effect of test agents on microbial population in secondary effluent from trickling filter process collected at two different dates.

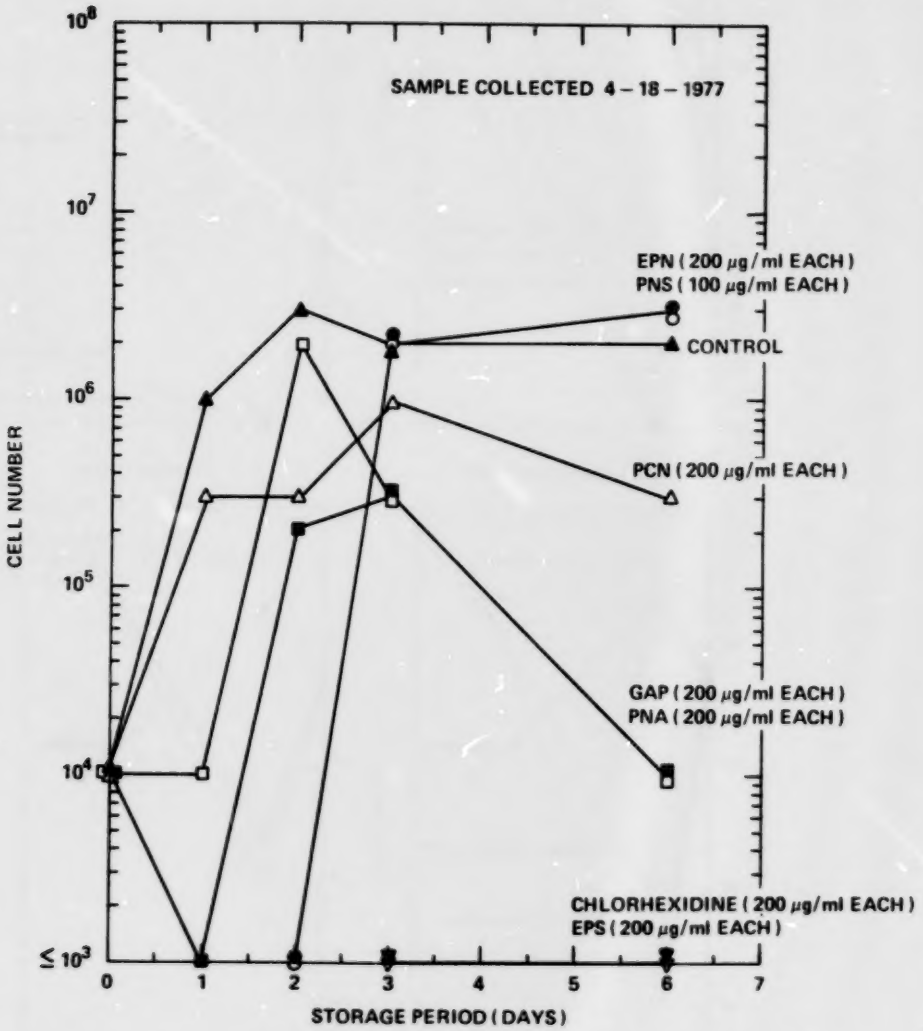

Figure 3. Effect of test agents on microbial population in secondary effluent from activated sludge process.

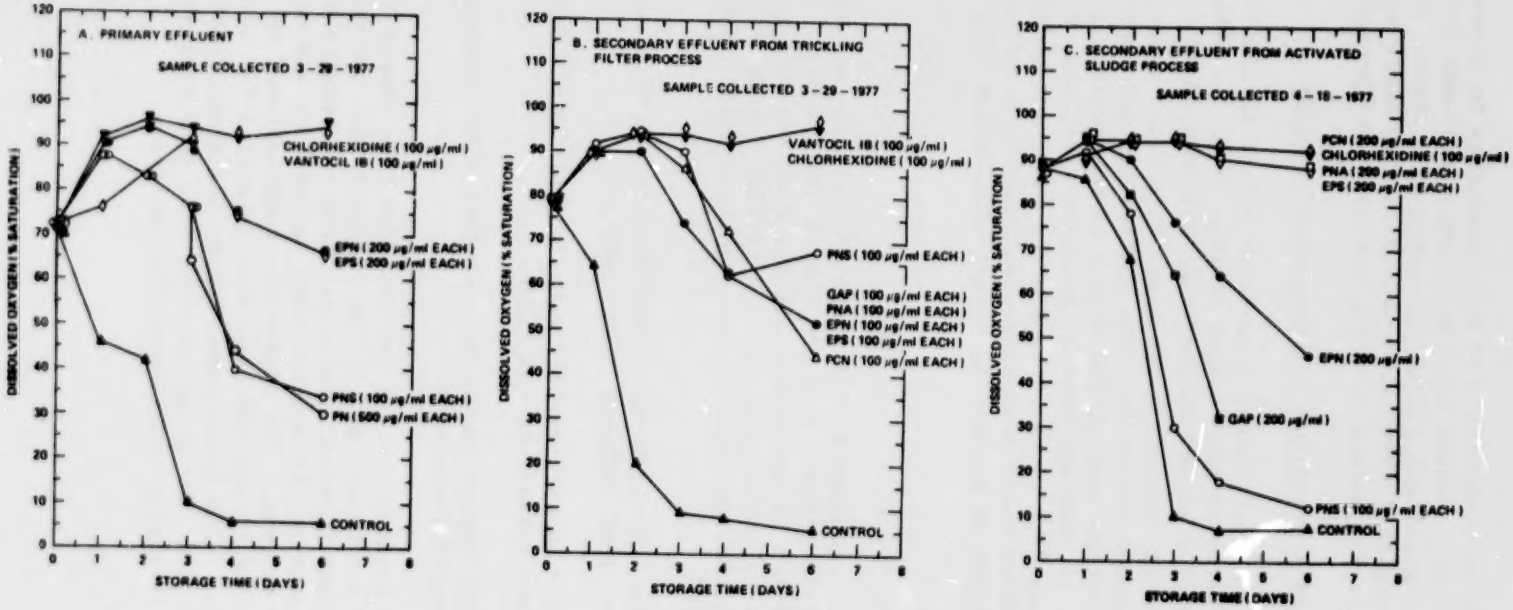
A. PRIMARY EFFLUENT
SAMPLE COLLECTED 3 – 29 – 1977
CHLORHEXIDINE (100 µg/ml)
VANTOCIL IB (100 µg/ml)
EPN (200 µg/ml EACH)
EPS (200 µg/ml EACH)
PNS (100 µg/ml EACH)
PN (500 µg/ml EACH)
CONTROL
DISSOLVED OXYGEN (% SATURATION)
STORAGE TIME (DAYS)
B. SECONDARY EFFLUENT FROM TRICKLING FILTER PROCESS
SAMPLE COLLECTED 3 – 29 – 1977
VANTOCIL IB (100 µg/ml)
CHLORHEXIDINE (100 µg/ml)
PNS (100 µg/ml EACH)
GAP (100 µg/ml EACH)
PNA (100 µg/ml EACH)
EPN (100 µg/ml EACH)
EPS (100 µg/ml EACH)
PCN (100 µg/ml EACH)
CONTROL
DISSOLVED OXYGEN (% SATURATION)
STORAGE TIME (DAYS)
C. SECONDARY EFFLUENT FROM ACTIVATED SLUDGE PROCESS
SAMPLE COLLECTED 4 – 18 – 1977
PCN (200 µg/ml EACH)
CHLORHEXIDINE (100 µg/ml)
PNA (200 µg/ml EACH)
EPS (200 µg/ml EACH)
EPN (200 µg/ml)
GAP (200 µg/ml)
PNS (100 µg/ml EACH)
CONTROL
DISSOLVED OXYGEN (% SATURATION)
STORAGE TIME (DAYS)

Figure 4. Dissolved oxygen as affected by the presence of test agents in primary and secondary municipal wastewater effluents.

known to enhance the susceptibility of microorganisms to lysozyme attack, was also without effect. Sodium sulfide in concentrations as high as 1000 ppm showed no significant effect on viable cell count. Oxygen concentration in the samples treated with sodium sulfide decreased rapidly. This may be linked to the strong reducing action of sodium sulfide.

Effect of Drugs on Microorganisms in Fresh Water Samples

All the drugs examined showed a positive effect in inactivating microorganisms in fresh water samples during storage (Fig. 5). Unlike the treated samples, control samples showed substantial increases in cell numbers. The drugs were effective at concentrations lower than those needed to control microorganisms in sewage effluents. The antibiotics and biocides became less effective when the waters were spiked with a carbon source (glucose-glutamic acid 150 mg/ℓ each) to simulate heavily polluted fresh waters (Fig. 5D). Vantocil was the only drug among those studied which resembled $HgCl_2$ in bacteriocidal action under these conditions, although both vantocil and chlorhexidine were effective in preventing oxygen utilization (Fig. 6).

CONCLUSION

Sewage effluents contain a wide range of bacteria, as well as a large population of bacteria (Taber, 1976 and Dias & Bhat, 1964). Natural water, unless heavily polluted by wastewater discharge or run-off, would not support such large populations of bacteria but may contain a wide variety of bacteria. Any antibiotics selected as possible preservatives should therefore meet several requirements:

1. Antibiotics should be broad spectrum in nature, i.e. effective against a broad range of microorganisms.

2. They should be sufficiently water soluble and stable to pH and temperature variations and light.

3. Antibiotics chosen should be such that microorganisms do not develop rapid resistance.

The amount of antibiotic that can be added to a sample is limited by the solubility of that antibiotic and by the prospect that too much antibiotic may interfere with any chemical analysis. From the results of the experiments presented in this section, it appears that vantocil IB and chlorhexidine are the best routes to explore. The antibiotic combinations: PNS, EPN, EPS, PCN, GAP and to some extent PN and PNS also show potential for water and wastewater sample preservation, since these drugs controlled microorganisms in many samples but not in all. The drugs selected here were evaluated for their ability to stabilize sample constituents/parameters.

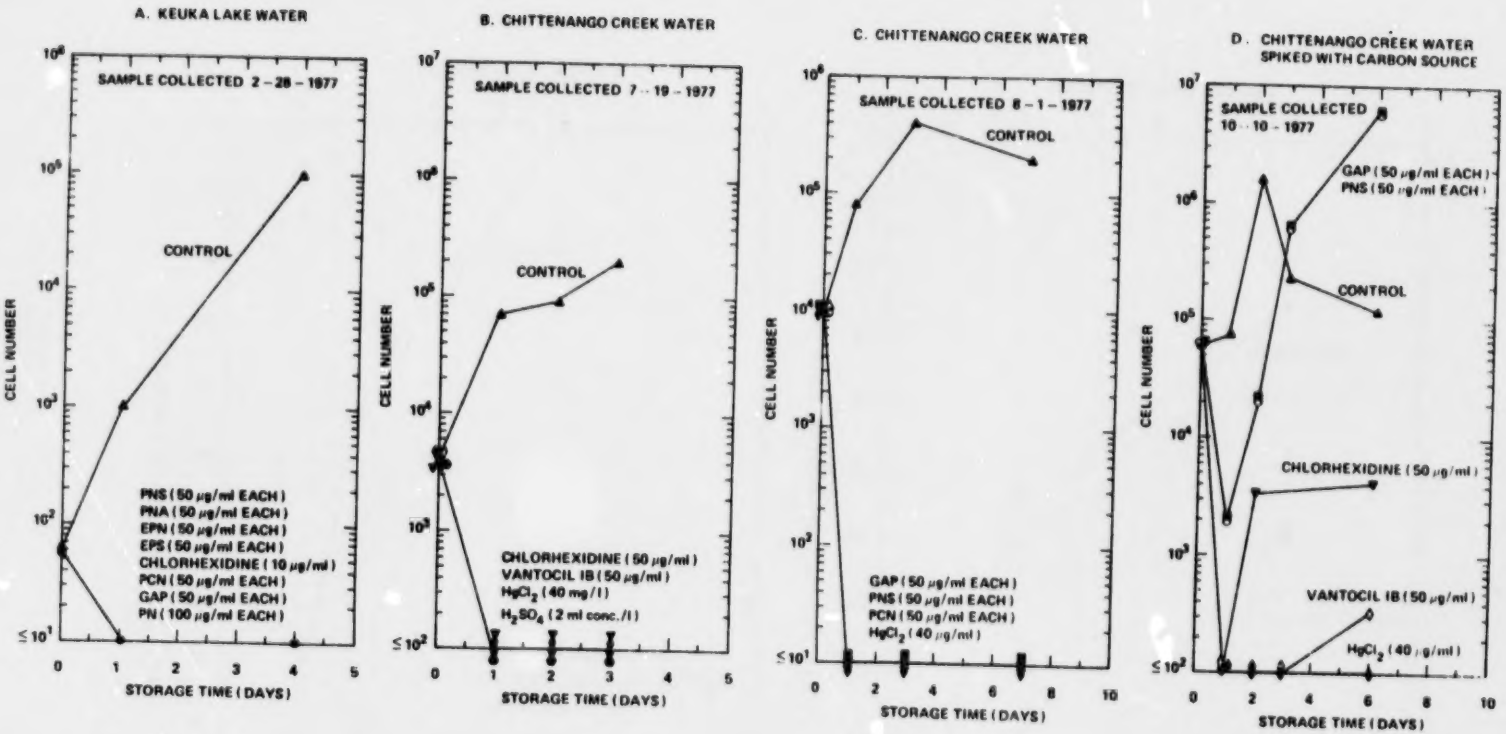
A. KEUKA LAKE WATER
SAMPLE COLLECTED 2 – 28 – 1977
CONTROL
PNS (50 µg/ml EACH)
PNA (50 µg/ml EACH)
EPN (50 µg/ml EACH)
EPS (50 µg/ml EACH)
CHLORHEXIDINE (10 µg/ml)
PCN (50 µg/ml EACH)
GAP (50 µg/ml EACH)
PN (100 µg/ml EACH)
CELL NUMBER
STORAGE TIME (DAYS)
B. CHITTENANGO CREEK WATER
SAMPLE COLLECTED 7 – 19 – 1977
CONTROL
CHLORHEXIDINE (50 µg/ml)
VANTOCIL IB (50 µg/ml)
$HgCl_2$ (40 mg/l)
H_2SO_4 (2 ml conc./l)
CELL NUMBER
STORAGE TIME (DAYS)
C. CHITTENANGO CREEK WATER
SAMPLE COLLECTED 8 – 1 – 1977
CONTROL
GAP (50 µg/ml EACH)
PNS (50 µg/ml EACH)
PCN (50 µg/ml EACH)
$HgCl_2$ (40 µg/ml)
CELL NUMBER
STORAGE TIME (DAYS)
D. CHITTENANGO CREEK WATER
SPIKED WITH CARBON SOURCE
SAMPLE COLLECTED
10 – 10 – 1977
GAP (50 µg/ml EACH)
PNS (50 µg/ml EACH)
CONTROL
CHLORHEXIDINE (50 µg/ml)
VANTOCIL IB (50 µg/ml)
$HgCl_2$ (40 µg/ml)
CELL NUMBER
STORAGE TIME (DAYS)

Figure 5. Effect of test agents on microbial population in natural waters.

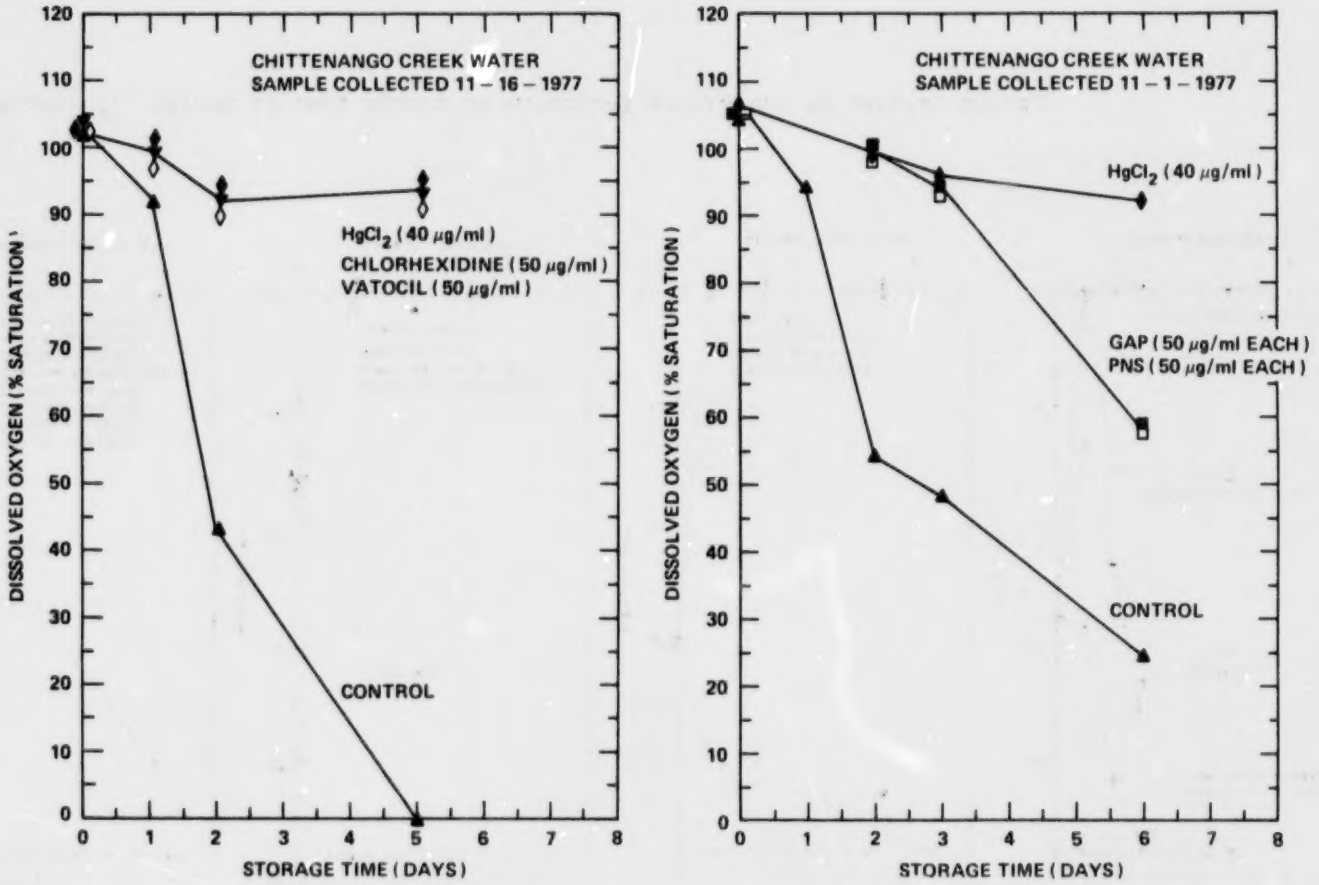

Figure 6. Dissolved oxygen as affected by the presence of test preservation agents in natural water samples fortified with glucose-glutamic acid (150 mg/ℓ each).

SECTION VII

EVALUATION OF SELECTED TREATMENTS AS PRESERVATIVES OF NITROGEN-FORMS IN WATER AND WASTEWATER

Nitrogen is present in water in several forms - organic nitrogen, ammonia, nitrate and nitrite. Since microorganisms present in water are capable of utilizing and/or causing interconversion of these forms, it is necessary to add an effective preservative to the sample at the time of collection. Howe and Holley (1969) reported the following changes in the untreated samples of surface water and settled sewage: (a) NH_3 concentration increased during early periods of storage but decreased to zero after 20 days at room temperature. (b) Organic nitrogen decreased rapidly within the first five days then stabilized. (3) NO_2 increased for the first few days, later decreased to zero, with a concomitant increase in NO_3. Similar changes in nitrogen-forms in water samples during storage at room temperature were noted by others (Hellwig, 1964, 1967).

No ideal preservative has yet been found which can be used to stabilize all nitrogen parameters without interference with analytical methods. Sulfuric acid is not a practical preservative when either NO_2 or NO_3 is to be examined, since acid catalyzes the conversion of NO_2 to NO_3 (Brezonik & Lee, 1966; Howe & Holley, 1969; Jenkins, 1968). Even preservation by $HgCl_2$ has its limitations. At the recommended concentration of 40 µg/ml, $HgCl_2$ does not preserve highly polluted samples (Hellwig, 1967). Higher concentrations are not recommended because of interference with the distillation step in the analysis for NH_3 (Hellwig, 1967; Huibregtse, 1976; and Howe & Holley, 1969). If NO_2 or NO_3 is not a parameter to be examined, then acidifying the samples to a pH of 2.0 with concentrated sulfuric acid would be a preferred method because of the health and environmental hazards linked with the use of $HgCl_2$. Freezing of the samples stabilizes NO_2, NH_3, NO_3, and Kjeldahl nitrogen but often immediate freezing of samples is impractical or impossible and there may be a delay of anywhere from several hours to several days before freezing (Marvin & Proctor, 1965; Proctor, 1962; Degobbis, 1973; and Thayer, 1970).

This section presents the results of experiments designed to test the effectiveness of antibiotics and biocides in preserving NO_2 and NO_3 levels in natural water and effluent samples. The objective was to find a suitable replacement to the currently used preservatives, which would be safe to use and would not interfere with any of the nitrogen-series analyses.

EXPERIMENTAL PROCEDURE

Primary and secondary effluents and fresh waters were collected as described earlier. Samples were aliquoted in 59 ml volumes in glass screw top bottles (capacity 60 ml). Following addition of 1 ml solution of the test drug, the bottles were stored at room temperature. Whole samples were sacrificed at designated intervals for analysis of nitrogen forms. Sample stored without any preservative served as a control. Several effluents, particularly primary effluents, had to be passed through a prefilter and a membrane filter (0.45 nm) prior to use in the preservation studies.

Nitrate was determined by the Brucine method and nitrite by diazotization method (USEPA, 1974). Ammonia was determined with an Orion ammonia electrode, Model 95-10, according to instructions in the accompanying instruction manual. Absorbance values were determined with a Bausch and Lomb Spectronic 20.

RESULTS AND DISCUSSION

Initial experiments were performed to determine which drugs, if any, would interfere with the chemical determination of nitrogen compounds in water. Standard solutions of nutrients were prepared and divided into two portions. Test preservative was added to one portion while the other served as control. Nutrient analyses were performed on the two samples and the values obtained were compared. The results are presented in Table 4. Several antibiotic combinations interfered with the Brucine determination of NO_3, especially combinations containing erythromycin or ampicillin. None of the combinations tested interfered with NO_2 determination by the diazotization method. Although no specific testing was done for interference with NH_3 determination, none was anticipated if the probe method of determination was used. The antibiotic combinations and biocides which showed no/or minimal interference were used for further studies.

Fresh waters collected from Chittenango Creek showed measurable concentrations of NO_3 (0.30 - 0.87 mg/ℓ) but very low concentrations of NO_2 ($\sim$0.014 mg/ℓ). The samples were therefore spiked with nitrite (0.017 mg/ℓ) prior to use. Evaluation of the prospective preservation agents could not be pursued with these waters because the concentration of nitrate and nitrite did not vary significantly upon storage even in the unpreserved samples. The problem was overcome by fortifying the samples with a carbon source (glucose-glutamic acid, 150 mg/ℓ each) to stimulate microbial activity. The addition of an exogenous carbon source induced a rapid decrease in the levels of NO_3 and NO_2 (Figs. 7 and 8). Chlorhexidine and vantocil (50 µg/ml) stabilized NO_3 and NO_2 in these samples for up to 30 days. PNS and GAP (50 µg/ml each antibiotic) could stabilize these nutrients only for 3 days. Increasing the drug concentration to 100 µg/ml of each antibiotic did not show a significant improvement in their preservation potential. Effort to retest these drugs on another fresh water source (Onondaga Lake water) failed because of the presence of a substance in this water which interfered with nitrate measurement. Onondaga Lake is heavily contaminated with industrial and domestic waste and therefore the interference noted during analytical measurement is not surprising.

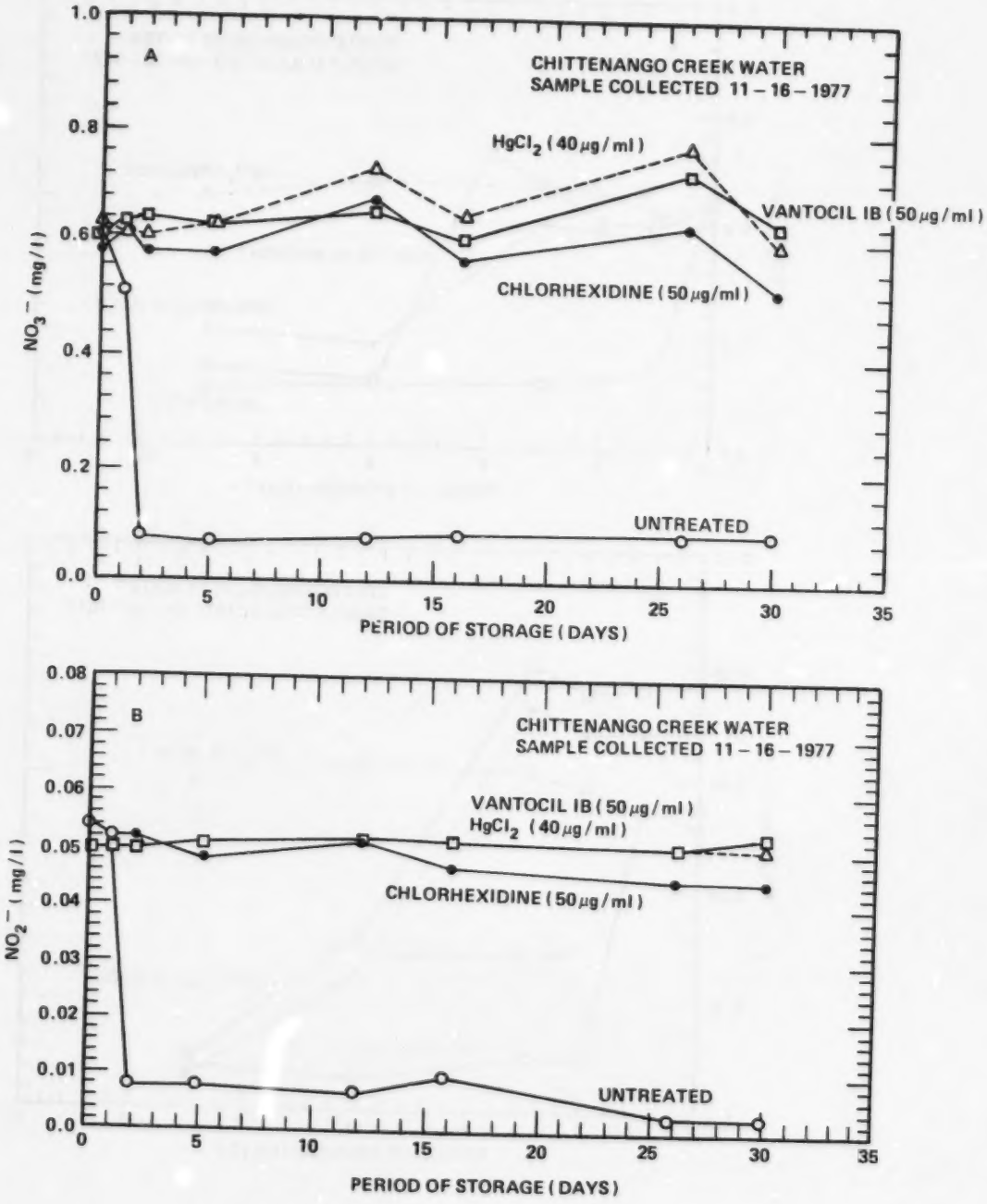

Figure 7. Preservation of nitrate and nitrite in spiked natural water samples by vantocil and chlorhexidine. Samples were fortified with glucose-glutamic acid (150 mg/ℓ each) to induce changes in the levels of nitrate and nitrite.

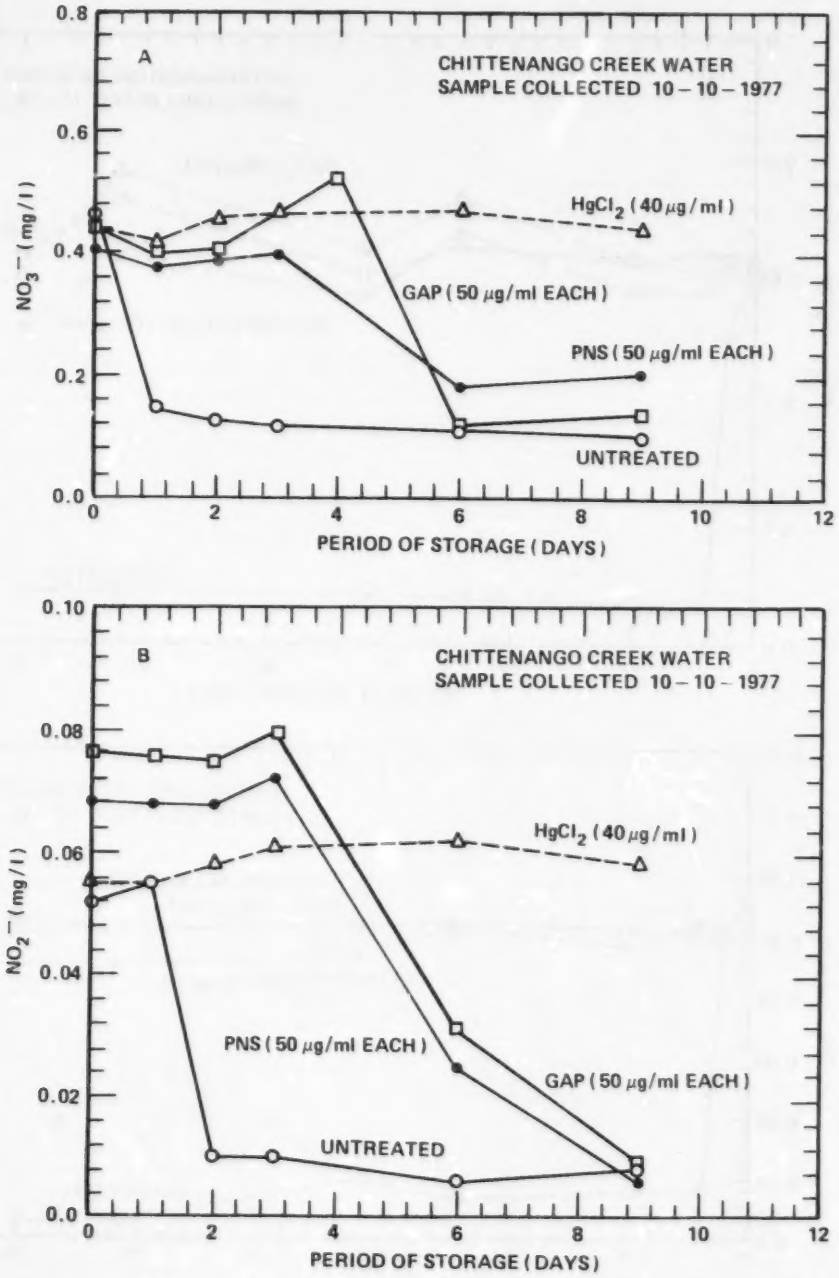

Figure 8. Preservation of nitrate and nitrite in spiked natural water samples by antibiotic combinations GAP and PNS. Samples were fortified with glucose-glutamic acid (150 mg/ℓ each) to induce changes in the levels of nitrate and nitrite.

TABLE 5. INFLUENCE OF THE PRESENCE OF PRESERVATIVES ON ANALYTICAL DETERMINATION OF NITRATE AND NITRITE

Treatment	Concentration (µg/ml)	Analytical Interference	
		NO_2	NO_3
Chlorhexidine	50 - 100	-	-
PNS	100	-	-
EPN	100 - 200	-	+
PCN	100	-	-
GAP	100	-	-
PN	500	-	-
PNA	100	-	+
EPS	200	-	+
Vantocil	100	-	-

+ Interference.
- No interference.

Storage of unpreserved sewage samples from activated sludge process at room temperature was accompanied with increase in NO_3 and NO_2 levels which continued up to 14 days (Figure 9). The levels of NO_3 remained unchanged afterward, while the level of NO_2 sharply declined. These nutrients were stabilized in the samples to which chlorhexidine or vantocil had been added at 100 µg/ml. In primary effluents, these drugs failed to stabilize NO_3 concentrations while the results with regard to NO_2 stabilization were inconclusive. PNS, the only antibiotic combination evaluated for sample preservation potential in trickling filter effluent, proved to be ineffective. Vantocil showed potential in stabilizing changes in NH_3 concentrations in effluent samples, however, confirmation of these findings is necessary.

CONCLUSION

Chlorhexidine and vantocil IB show promise as preservatives of NO_2 and NO_3 levels in fresh water, and relatively clean sewage effluent samples. They offer no interference with the analytical determination of NO_2 and NO_3. The biocides are effective for at least 28 days of storage at room temperature. If these treatments are combined with storage at 4°C, holding times might be extended.

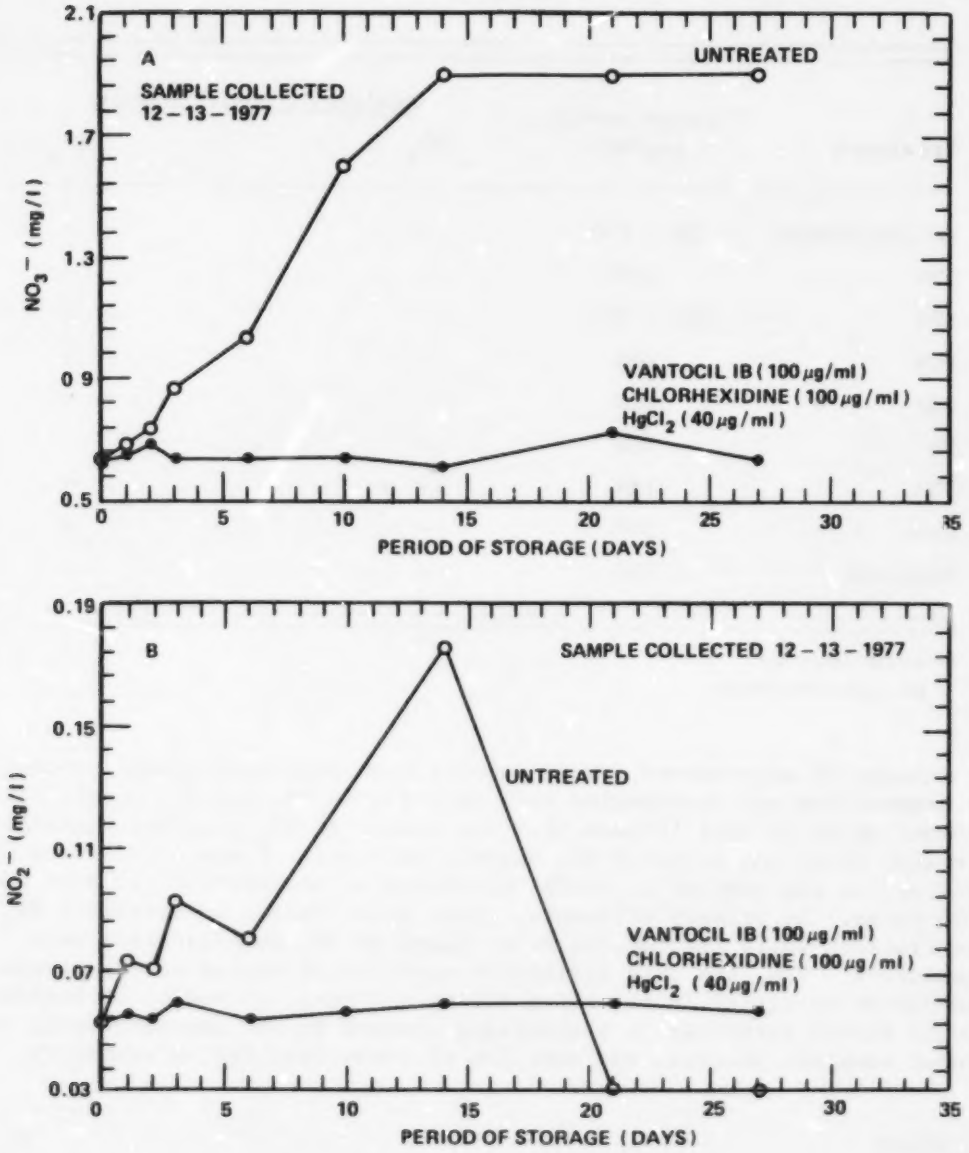

Figure 9. Preservation of nitrate and nitrite secondary effluent from activated sludge process.

Antibiotics and biocides at the concentration range of 100-200 µg/ml fail t preserve highly polluted samples (e.g. primary effluent). Use of concentrations higher than these is impractical because of solubility problems and possible interference with analytical determinations.

SECTION VIII

EVALUATION OF SELECTED TREATMENTS AS PRESERVATIVES OF PHOSPHATE IN WATER

INTRODUCTION

The measurement of phosphate is an integral part of water and wastewater analysis since high levels of phosphate can seriously disrupt the ecology of water systems. In freshwater ecosystems, an increase in N and P compounds can accelerate the complex series of interactions which govern the growth of plant, animal, and microbial populations. Increase in concentration of nutrients can result in the increased growth of littoral vegetation, development of algal blooms, and deoxygenation of hypolimnic waters (Keeney, et al., 1971).

If samples are stored without treatment, marked changes in the level of inorganic and organic phosphate can occur due to microbial metabolism, and/or adsorption on detritus and sample bottle walls (Gilmartin, 1967). The level of inorganic phosphate may increase by enzymatic decomposition of organic phosphates or it may decrease as bacteria, in the process of multiplication, convert inorganic phosphate to organic phosphate cellular compounds and components (Murphy & Riley, 1956).

Phosphate preservation involves many variables, including size and material of sample container, volume and type of sample material, filtration, length and temperature of storage, concentration and form of preservative, concentration range of phosphate studied, and the methods of analysis employed (Jenkins, 1967). Numerous chemical preservatives have been tested, including chloroform, toluene, 1,2 dichloroethane, sodium fluoride, aluminum hydroxide, thymol, formalin, mercuric chloride, and sulfuric acid (Hellwig, 1964; Heron, 1962; Gilmartin, 1967; Jenkins, 1967; and Murphy & Riley, 1956). Addition of $HgCl_2$ or H_2SO_4 along with cooling to 4°C is the method currently recommended by EPA for preservation of various forms of phosphate in water and wastewater samples for up to 24 hours (U.S.EPA, 1974). H_2SO_4 is not recommended for preservation of condensed phosphates due to their instability in acidic samples. $HgCl_2$ may cause analytical interference when the chloride level of the sample is low.

Algae can contain large quantities of phosphorus and their death by the added preservative can result in release of significant quantities of phosphorus in the sample. To overcome this problem, Fitzgerald and Faust (1967) recommended that samples intended for preservation by methods other than refrigeration be filtered to remove algae.

The review of literature presented above shows that there is need for developing more effective and safer method for stabilizing various forms of phosphates in water and wastewater samples. This section presents the results of several experiments designed to test the effectiveness of antibiotics and chemical biocides in the preservation of phosphates.

MATERIALS AND PROCEDURE

Fresh water and wastewater samples were collected as described in Section VI. Experimental procedure for phosphate preservation studies was similar to that described earlier for preservation of nitrogen compounds. Fresh waters were spiked with potassium dihydrogen phosphate to give 0.1 mg P/ℓ prior to use in preservation studies. Effluent samples which contained large amounts of suspended material were filtered through a glass fibre prefilter and 0.45μ filter. Membrane filters were prewashed by soaking overnight in distilled water (APHA, 1975).

Phosphate was determined using the EPA recommended Ascorbic Acid Method (U.S. EPA, 1974). Absorbance values were determined with Bausch and Lomb Spectronic 20.

RESULTS

Initial studies were performed to determine which antibiotics/chemical biocides would interfere with the chemical determination of phosphate by the Ascorbic Acid and Vanadophosphoric Acid Methods. A standard solution of orthophosphate (0.2 mg P/ℓ) was divided into two portions - test preservative was added to one half, the remainder was without any additions. Analyses were performed on the two samples and the values obtained were compared.

Orthophosphate could not be determined in the presence of EPS or vantocil by the Ascorbic Acid Method (Table 6). The latter biocide resulted in the formation of a white precipitate which precluded absorbance measurement. Other antibiotics presented no interference in the concentration range tested.

All the antibiotics/chemical biocides listed in Table 6 interfered with orthophosphate determination by Vanadophosphoric Acid Method (APHA, 1975). A precipitate was formed upon the addition of color reagent which precluded making absorbance measurements. Interference caused by the presence of PNA, EPS and vantocil persisted even after the persulfate digestion of samples required for total phosphate determination.

Preservation studies with fresh waters were conducted using samples obtained from Chittenango Creek. Spiked untreated samples during 10 day storage at room temperature failed to show marked changes in orthophosphate- and total phosphate concentrations. Changes were induced by addition of glucose-glutamic acid (150 mg/ℓ each) to the samples (Fig. 10). Orthophosphate levels could not be stabilized by GAP or PNS (50 μg/ml each antibiotic), or by $HgCl_2$. No improvement in preservation ability was seen upon increasing

the antibiotic concentration to 100 µg/ml for each antibiotic. Chlorhexidine interfered with orthophosphate analysis in many fresh water samples and hence could not be studied.

Difficulties were encountered in preservation of phosphates in sewage samples. Many antibiotics/chemical biocides which presented no interference in phosphate determinations in spiked distilled water caused severe interference in sewage samples. The intensity of interference was somewhat dependent upon the quality of the samples. For example, interference was most intense and occurred frequently in primary effluent samples. Filtration of sample through 0.45µ filters prior to analysis failed to remove the interfering material.

TABLE 6. EFFECT OF THE ADDITION OF TEST SAMPLE PRESERVATION AGENT ON ORTHOPHOSPHATE DETERMINATION BY ASCORBIC ACID METHOD

Antibiotic/chemical biocide added	Concentration (µg/ml each)	Effect noted
PNS	50 - 100	-
PNA	100	*
EPS	100 - 200	+
EPN	50 - 100	-
PN	500	*
GAP	50 - 100	-
PCN	50 - 100	-
Vantocil IB	50 - 100	+
Chlorhexidine	100	-

- = No interference, + = Interference, * = Not determined.

CONCLUSION

The antibiotics and chemical biocides tested do not offer a solution to the problem of phosphate preservation in water and wastewater samples. Most test agents interfered with phosphate analysis and those which did not interfere were ineffective in preserving samples for any reasonable length of time. Combining preservation action of antibiotics/chemical biocides with storage at 4°C may offer hope for preserving these samples.

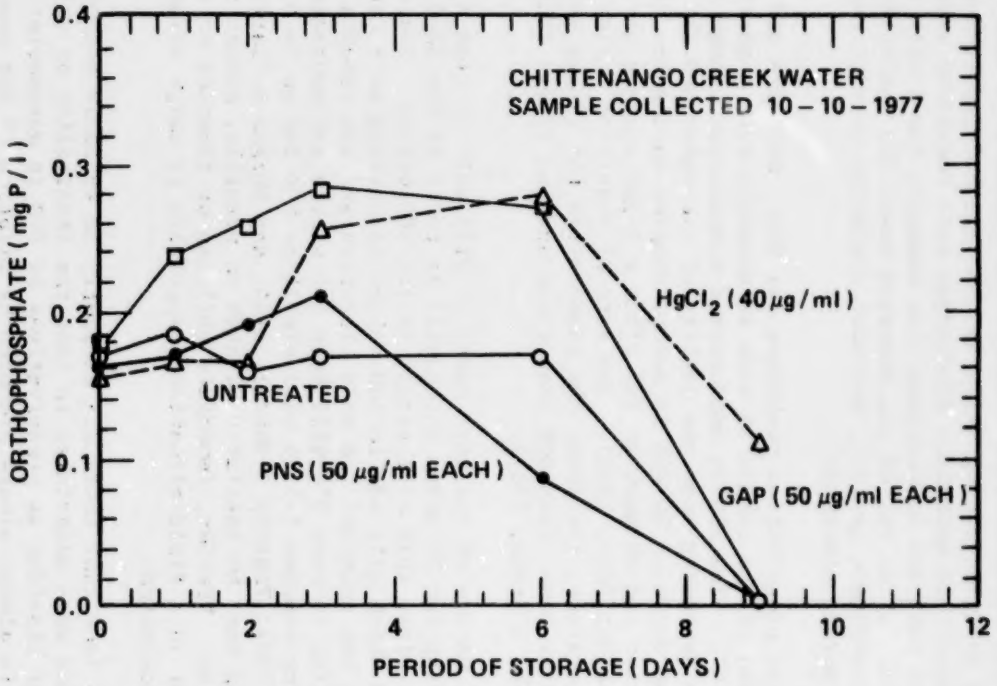

Figure 10. Influence of antibiotics additions on the levels of orthophosphate in spiked fresh waters. The samples were fortified with glucose-glutamic acid (150 mg/ℓ each) to induce changes in phosphate concentration.

SECTION IX

EVALUATION OF SELECTED TREATMENTS AS PRESERVATIVES OF BOD IN WASTEWATER

Biochemical oxygen demand (BOD) has been used as a measure of the presence of organic materials in aqueous solution and, subsequently, as a measurement of water quality. BOD determination reflects the presence of materials in water which will be oxidized biologically and has special importance in determining the quantity of materials discharged into receiving water (Stack, 1971). Although the test has limitations (for example, the length of time required for testing), it is one of the standard tests for evaluation of water quality, especially effluent quality, and most discharge permits require BOD monitoring to meet permit standards.

There is no real preservation technique for BOD. Both EPA and the APHA recommend utilization of the sample as soon as possible with a maximum holding time of 6 hours at 4°C. The BOD in unpreserved samples can change dramatically in very little time depending upon the quality of the water and the holding conditions. BOD can decrease 20-40% in sewage samples during the first 6-8 hours after collection and as much as 90% after a 3-day storage at room temperature (Phillips & Hatfield, 1941). The rate of reduction varies according to the storage temperature, the lower the temperature the slower the rate, and the sealed samples lose less BOD than those exposed to air (Phillips & Hatfield, 1941; Loehr & Brown, 1967).

The biological nature of the test makes it difficult to find a suitable preservative. Nothing can be added which will be toxic to the seed employed in the actual test, since this will result in low BOD values. Likewise, nothing can be used which will add to BOD values by serving as a carbon source. Temperature control has received the most attention and the results of testing indicate that chilling to near 0°C will keep BOD stable for approximately 6 days while freezing samples (-5°C) will stabilize BOD for up to 6 months (Loehr & Bergeron, 1967; Fogarty & Reeder, 1964; and Morgan & Clark, 1964). Chilling or freezing must be initiated as soon as possible, either during or soon after collection. However, immediate chilling or freezing of samples may not be practical in a field situation, especially if large volumes of sample need to be collected.

In view of the fact that there are currently no suitable methods for BOD preservation, studies were undertaken to test the feasibility of using antibiotics and chemical biocides as preservatives of BOD in wastewater samples. Since BOD analysis is almost always preceded by dilution of the samples, it was assumed that dilution would relieve the antimicrobial action of the preservative, thereby making BOD measurement possible. In addition, several

antibiotics which are known to be bacteriostatic as opposed to bacteriocidal were also examined for their ability to preserve BOD. Such antibiotics do not irreversibly damage microorganisms, and therefore they may be more suited for this purpose.

MATERIALS AND PROCEDURE

These studies were carried out using secondary effluents from the Meadowbrook-Limestone Wastewater Treatment Plant. Samples were collected and brought to the laboratory as described in Section VI. BOD's of these wastewaters ranged between 3.45 - 19.1 ppm during the study period. Samples were stored in glass bottles at room temperature with or without the addition of preservative. Whole samples were sacrificed for BOD measurement at desired intervals. Experiments were performed in triplicate and results are expressed as averages.

BOD determinations were performed according to the procedure in Standard Methods for the Examination of Water and Wastes, 14th ed. Settled sewage was used as seed and added to dilution water at a concentration of 2 ml/ℓ. A standard solution containing 150 mg/ℓ each of glucose and glutamic acid was included with each BOD study as a check for seed viability and experimental procedure as recommended by APHA. Dissolved oxygen was measured with a YSI Model 54ARC Dissolved Oxygen Meter with BOD probe and attached stirrer.

RESULTS AND DISCUSSION

Presence of antimicrobial agents in the samples intended for BOD analysis can present the following problems: (i) sample dilution for BOD determination may not be enough to overcome antimicrobial action of the drug; this will result in inhibition of the seed activity and consequently in low BOD values; (ii) the antimicrobial agents may serve as an additional carbon source leading to higher BOD values for treated samples. It was essential, therefore, to study the interference of such agents with BOD analysis prior to evaluating their preservation potential.

Test preservatives were added to freshly collected wastewater samples and 5-day BOD was determined following the appropriate dilutions (1:5 to 1:20). The results were compared with the BOD of untreated samples. The antimicrobial drugs studied included chlorhexidine, vantocil, PNS, and GAP. These drugs in the concentration range 10 - 100 µg/ml (before dilution for BOD) severely inhibited microbial metabolism, which led to very low or no biological oxygen demand. Preacclimation of the seed with test drugs failed to relieve the inhibition since the BOD remained between 0-6% of the control values. Of special interest was the finding that chlorhexidine caused complete inhibition of oxygen utilization at 10-20 µg/ml but only partial inhibition of oxygen utilization at 30-100 µg/ml. Oxygen depletion at higher concentration but none at lower concentration is difficult to explain. A possible explanation may be that a higher concentration of the drug induces appropriate enzymes which enable the microorganisms present to utilize the drug as a carbon source. The severe interference of these antimicrobial agents with BOD analysis precluded their utilization in preservation of samples for BOD.

Elimination or inactivation of the antibiotic prior to BOD analysis was explored as a possible solution to the problem. Studies with PNS (50-100 µg/ml) showed that boiling the drug solution in a sealed container for up to 40 minutes failed to affect the potency of the drug. The drug was stable to alkaline and acid conditions since pH adjustment was also without any effect. Autoclaving caused a significant decrease in the BOD_5 of untreated samples and was, therefore, not given further consideration.

It appeared likely that drugs which are bacteriostatic as opposed to bacteriocidal may offer a better chance for stabilizing BOD samples. Two bacteriostatic antibiotics - chloramphenicol and erythromycin - were studied for their preservation potential. Chloramphenicol did not significantly affect BOD_5 values when added to the wastewaters at concentrations up to 10 µg/ml. Higher drug concentrations gave lower BOD_5 values suggesting inhibitory effect of the drug. Erythromycin at similar concentrations (10 - 50 µg/ml) caused complete inhibition of oxygen depletion. Chloramphenicol at concentrations lower than 10 µg/ml failed to prevent microbial growth in wastewaters during storage and therefore was considered of little value as a sample preservation agent. Furthermore, utilization of this drug by microorganisms as a carbon source at these concentrations also presented a problem. BOD_5 of a pure antibiotic solution (10 µg/ml before BOD dilution) varied from time to time and ranged between 1.6 - 5.0 ppm

BOD preservation could not be achieved even by storage of chloramphenicol (<10 µg/ml) treated samples at 4°C. Combining antibiotic action with adjustment of the sample to a nonphysiological pH (pH 4.0 or 10.)) was not considered for BOD_5 preservation because pH adjustment by itself caused a significant lowering of the BOD_5 of the samples.

CONCLUSION

The biological nature of the BOD test makes it very difficult to find a suitable method for BOD preservation. Chemical additives cannot be used since they may prove to be toxic to microorganisms even after the necessary dilution for BOD analysis. Any additive that can be metabolized by microbes cannot be used since it will contribute to BOD of the sample.

No suitable method for preservation of samples for BOD analysis is currently available. EPA recommends storage of samples by refrigeration but only for a maximum period of 6 hours. Freezing is a generally recognized method for long term preservation (Loehr & Bergeron, 1967; Fogarty and Reeder, 1964; and others). Freezing reduces the microbial activity to a minimum without irreversibly damaging the microorganisms. It was considered feasible to achieve the same objective by addition of bacteriostatic drugs. At the time of BOD analysis, it was hoped that sample dilution would relieve the static effect, thereby allowing interference-free BOD analysis.

In this study, antimicrobial drugs could not be successfully used to preserve samples intended for BOD analysis. The problem was related to the biological nature of the BOD test. The drugs tested interfered with BOD analysis by inhibiting seed activity. Acclimation of seed to the drug did not alleviate the problem. Lower concentrations of the drugs failed to preserve samples. In some instances they served as carbon sources and led to erroneous BOD_5 values. Selective elimination or inactivation of the drug prior to BOD_5 analysis could not be achieved by pH adjustment or heating of the treated sample.

SECTION X

EVALUATION OF SELECTED TREATMENTS AS PRESERVATIVES OF OIL AND GREASE IN WASTEWATER

INTRODUCTION

Oil and grease may be present in water due to the decomposition of planktonic and/or higher forms of aquatic life, and from pollution resulting from industrial and domestic wastes (Hach Chemical Co., 1975). Since many of these hydrocarbons can be utilized by bacteria, it is essential that samples to be analyzed for oil and grease be stored properly. The accepted procedure is to acidify samples to pH <2 and store at 4°C. Holding time is approximately 24 hours (U.S. EPA, 1974). We examined the possibility of long-term preservation of oil and grease samples by addition of antibiotics and chemical biocides.

MATERIALS AND PROCESURES

Effluent samples were collected from the overflow weirs of clarifiers at Wetzel Road and Meadowbrook-Limestone Treatment Plants as described in Section VII. Samples were brought to room temperature prior to use. One liter volumes were distributed into 1 liter, glass-stoppered bottles. Following the addition of the desired preservatives they were stored at room temperature. Whole samples were sacrificed for analysis. Oil and grease content was determined by extraction with petroleum ether (APHA, 1971).

RESULTS AND DISCUSSION

Preliminary experiments were performed to determine which antibiotics would interfere with oil and grease analysis. Effluent samples were divided into two portions. One remained without any additions, test drugs (100-200 µg/ml each antibiotic) were added to the other. Oil and grease determinations in the two samples revealed no interference by the drugs tested - chlorhexidine, vantocil, GAP, PNS, EPN, and PCN.

PCN and GAP were found to be effective in preserving oil and grease in secondary effluents obtained from activated sludge process for 8-12 days (Fig. 11). Other drugs exhibited no effect on the changes occurring in oil and grease content during sample storage. PCN and GAP could not stabilize oil and grease in primary effluents or in secondary effluent obtained from trickling filter process. The results show that antibiotics can effectively stabilize oil and grease in relatively clean effluents only. If the antibiotics are used along with storage at 4°C, it may be possible to stabilize oil and grease in heavily polluted samples, and perhaps also to extend the holding period.

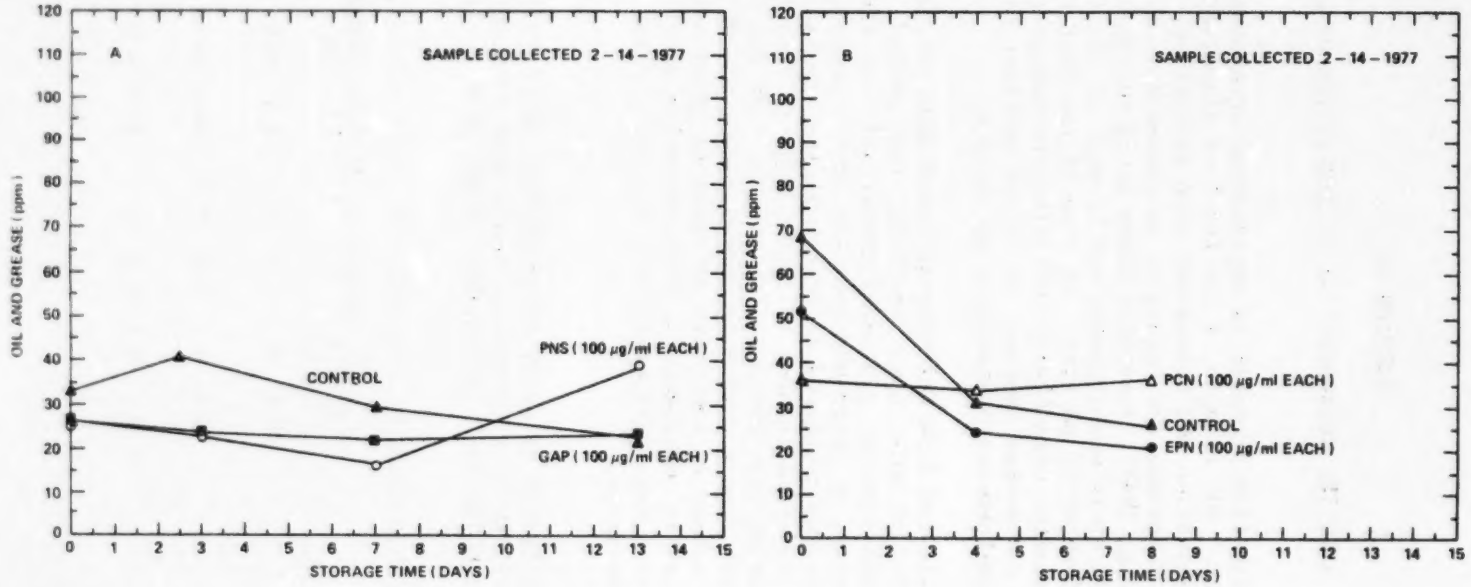
A
SAMPLE COLLECTED 2 – 14 – 1977
OIL AND GREASE (ppm)
STORAGE TIME (DAYS)
CONTROL
PNS (100 µg/ml EACH)
GAP (100 µg/ml EACH)
B
SAMPLE COLLECTED 2 – 14 – 1977
OIL AND GREASE (ppm)
STORAGE TIME (DAYS)
PCN (100 µg/ml EACH)
CONTROL
EPN (100 µg/ml EACH)

Figure 11. Potential of antimicrobial agents for preservation of oil and grease in the secondary effluent from activated sludge process.

SECTION XI

EVALUATION OF SELECTED TREATMENTS AS PRESERVATIVES OF pH IN WATER

As microbial metabolism proceeds in unpreserved aqueous samples, the pH of the samples changes. For example, a low level of dissolved oxygen may trigger anaerobic respiration and subsequent acid production, or the decomposition of nitrogenous compounds may result in an increase in pH (Loehr and Bergeron, 1967; Hellwig, 1964). Generally there is no recognized need for stabilizing pH changes since measurements can be made in the field with the help of a portable pH meter or pH kits. In view of the fact that many of the drugs tested in our studies effectively controlled microbial growth and oxygen utilization in water and sewage samples, it was of interest to determine if these agents would simultaneously stabilize pH changes.

Fresh waters collected from Chittenango Creek upon storage showed a pH change no more than 0.2 pH units over the 10-day test period. To accelerate a change in pH in these samples, glucose-glutamic acid was added (150 mg/ℓ each). In the fortified samples, pH decreased over the 30-day period at room temperature while it remained unchanged in chlorhexidine and vantocil treated samples (Table 7). The effectiveness of these drugs in stabilizing pH changes was also observed with Onondaga Lake water. PNS and GAP could not stabilize pH for longer than 6 days at a concentration of 100 µg/ml. Addition of mercuric chloride caused an immediate decrease in pH of some samples (Table 7) and is therefore unsuitable as a preservation agent for pH. Stabilization of pH in sewage effluents could not be achieved to a significant extent by any of these drugs.

TABLE 7. STABILIZATION OF pH BY ANTIMICROBIAL DRUGS IN FRESH WATER SAMPLES FORTIFIED WITH GLUCOSE-GLUTAMIC ACID (150 mg/ℓ each). SAMPLES COLLECTED FROM CHITTENANGO CREEK, DATE 11-16-77.

Test drug and concentration	Sample pH at days shown							
	0	1	2	5	12	16	26	30
Untreated	8.0	8.1	7.1	6.3	6.1	6.4	6.4	6.6
Chlorhexidine, 50 µg/ml	8.1	8.1	8.1	8.2	8.1	8.2	8.1	8.2
Vantocil IB, 50 µg/ml	8.1	8.1	8.1	8.1	8.1	8.2	8.1	8.2
$HgCl_2$, 50 µg/ml	7.4	7.4	7.5	7.4	7.4	7.5	7.6	7.8

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